(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number
WO 2004/022105 A2

(51) International Patent Classification[7]: A61K 51/04, 51/12, C07F 1/08, 13/00, 15/00

(21) International Application Number: PCT/US2003/027665

(22) International Filing Date: 2 September 2003 (02.09.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02078743.8 3 September 2002 (03.09.2002) EP

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/022105 A2

(54) Title: PREPARATION OF $M(CO)_3$-COMPLEXES BY SOLID PHASE TECHNIQUES VIA METAL ASSISTED CLEAVAGE FROM THE SOLID SUPPORT

(57) Abstract: The present invention relates to a process for generating a water soluble metal complexed agent, comprising contacting a solid phase bound organic conjugate represented by the formula I with $|M(H_2O)_3(CO)_3|^{n+}$, under suitable conditions to cause the formation of a coordinate bond between $|M(H_2O)_3(CO)_3|^{n+}$ and the tertiary amine nitrogen atom of the solid phase bound organic conjugate and thereby the release of the metal complexed agent thus formed from the support. The invention further relates to the conjugate of formula (I) and to a kit for performing the process.

PREPARATION OF $M(CO)_3$-COMPLEXES BY SOLID PHASE TECHNIQUES VIA METAL ASSISTED CLEAVAGE FROM THE SOLID SUPPORT

The invention relates to the field of radiopharmaceuticals. In particular the invention relates to a process for the preparation of a metal complexed agent via metal assisted cleavage from a solid support.

In a further aspect the invention relates to new solid phase bound conjugates of a ligand and a biomolecule.

In yet a further aspect the invention relates to new metal complexed ligand-biomolecule conjugates, compositions comprising these new complexes and their use.

In still a further aspect the invention relates to a kit for the preparation of a diagnostic or therapeutic pharmaceutical composition.

For the application of radiolabeled bioactive molecules such as i.e. peptides in clinical routine diagnosis or therapy it is highly desirable that only labeled compounds are injected to avoid saturation of the corresponding receptors *in vivo* or toxic side effects from "cold", unlabeled compounds. Furthermore, binding of large amounts of unlabeled biomolecules to the receptors spoils the possibility of getting clear images (scintigrams) and, thus, often disables a clear diagnosis.

According to the state of the art, high specific activity in a normal homogenous labeling procedure can only be achieved by using the lowest possible amount (concentration) of derivatized biomolecules (or ligand for ^{99m}Tc which is coupled to the biomolecule) which still results in quantitative labeling. Depending on the ligand and the complex

precursor, these amounts often have to be relatively high since at low concentrations the rate of complexation is governed by a second order kinetic and, thus, labeling is too slow and accompanied by decomposition of ligand or ^{99m}Tc precursor. The lowest concentration limit is often not convenient in routine use, since slightly changed conditions (temperature, time) at such a concentration do not end up with quantitative labeling yield. Correspondingly, side- and decomposition products as well as starting materials are still present in the final solution.

A convenient way of a physically separating 'cold' from 'hot' compound is by attaching the ligand-biomolecule conjugate to a solid phase material and cleave it from there concomitantly with the complex formation. Examples for such metal assisted cleavage from solid phases are rare.

American patent US-5,789,555 (Pollak et al.) describes a process for labeling peptides with technetium-99m, rhenium-186 or rhenium-188. The process comprising the steps of covalently coupling the peptides to a solid support, by means of a thioether bond with a maleimide linker. By introducing pertechnetate to the support, a $^{99m}Tc^{V}(=O)$-peptide complex is formed. Upon complex formation, $^{99m}Tc^{V}(=O)$ catalyzes cleavage of the peptide from the support, by breaking the C-S bond, thus releasing the $^{99m}Tc^{V}(=O)$-peptide complex from the support.

It is known from literature, that protected thiols release the protecting group by coordination to a Tc=O center. Based on these findings Pollak et. al. (J. Am. Chem. Soc. 121, 11593-11594 (1999) bound a tetradentate chelator via a thioether bond to a gold surface. Upon coordination of Tc(V)

to this ligand the ^{99m}Tc-complex was selectively released into solution by breaking the S-Au-bond as the sulfur coordinated to the Tc.

More recently, Dunn-Dufault, et. al. (Nucl. Med. Biol. 27, 803-807 (2000)) described a variant of this method by covalently binding the chelator to an organic polymeric support.

The above mentioned processes for producing Tc and Re labeled organic complexes all depend on cleavage of a C-S or Au-S bond. This C-S and Au-S bond, with which the ligand is covalently bound to the solid support, is sensitive to oxidation. Therefore, it is necessary to store solid supports comprising ligands covalently linked via a C-S bond under reducing conditions. This is especially true for long term storage. The necessity of storage under reducing conditions requires additional measures to be taken for storage. Moreover, if the supports are to be used for the generation of compounds suitable for pharmaceutical applications, the presence of reducing agents is highly undesirable from the standpoint of pharmaceutical safety. Therefore, there will be certain restrictions for use of the known solid bound ligands for such applications.

Additionally, the use of these metal oxide species is accompanied by restrictions to the ligands that are available for use therewith i.e. tetradentates. Hence the sole disclosure in the prior art of peptidic ligands for use with a $^{99m}Tc^{V}(=O)$ center.

Thus there is a need for new processes for preparing metal labeled complexes by solid phase techniques via metal assisted cleavage from the solid support which employ solid

phase bound biomolecule-ligand conjugates which are more stable under pharmaceutically acceptable conditions than the prior art conjugates.

Additionally, the availability of more ligands that can be used in the formation of metal complexed ligand-biomolecule conjugates by means of solid phase techniques via metal assisted cleavage will be advantageous, since this will provide a more flexible use of this technique. It is the object of the present invention to provide improved techniques for the preparation of labeled diagnostic and therapeutic compounds.

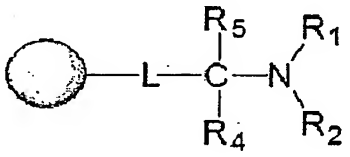
In the research that led to the present invention, it was found that some organic molecules (ligands) that are able to coordinate to a metal and bound to a solid support via a tertiary amine group, in the presence of $[Tc(H_2O)_3(CO)_3]^+$, cleave from the solid support upon formation of $[Tc(CO)_3$-Ligand]-complexes. The selective hydrolytic C-N bond cleavage is clearly mediated by the low valent carbonyl $[Tc(H_2O)(CO)_3]^+$ center, formed during complex formation, and does not occur under the same reaction conditions in the absence of $[^{99m}Tc(H_2O)_3(CO)_3]^+$. After release from the solid support, the former tertiary amine is present as a coordinated secondary amine.

The mechanism for this $[Tc(CO)_3$-Ligand]-complex is proposed to be as follows. As the tertiary amino group of the solid-bound chelator (the so-called ligand) coordinates to the cationic metal center of $[M(H_2O)_3(CO)_3]^{n+}$, it becomes partially positive and the adjacent carbon atom is therefore activated for nucleophilic attack. A remaining hydroxy group attacks the methylene group of the chelator and induces C-N bond cleavage

(Figure 2). The third donor site of the chelator coordinates to the metal center, and the product complex is released into solution. Uncomplexed chelator and uncleaved complex remain bound to the solid phase.

It was found that labeled compounds obtained by hydrolytic cleavage of the ligand from the solid support with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ as described above had a very high specific activity i.e. there was little uncomplexed ligand in solution. The amount of uncomplexed ligand in solution was in the order of 10^{-7} M. Therefore, this specific cleavage reaction can be attractively exploited for the preparation of so-called "no carrier added" (n.c.a.) complexes of technetium and other metals with a similar chemical reactivity.

Thus the invention relates to a new process for generating a metal complexed agent, comprising contacting (I) a solid phase bound organic conjugate represented by the formula



(I)

wherein:

the sphere is the solid phase;

C is a methylene group that may be substituted by one or two groups R4 and R5, which can be in particular aliphatic or

aromatic substituents, or RO, RS or R_2N, wherein R is an aliphatic or arylic group,

L is a linker that may or may not be present, that is coupled to the solid support and has activating properties towards nucleophilic attack to the C group and is preferably a phenyl, alkyl, allyl or aryl; and

R1 and R2 are the same or different and are a metal coordinating group or a non-coordinating organic group,

which solid phase bound organic conjugate is optionally derivatized at one or both of R1 and R2 with a biologically active molecule,

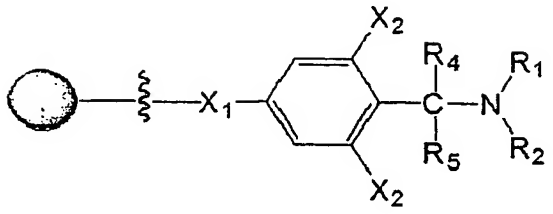
with (II) $[M(H_2O)_3(CO)_3]^{n+}$,

wherein M is selected from the group consisting of technetium (Tc), rhenium (Re), rhodium (Rh), platinum (Pt), iridium (Ir) and copper (Cu) and n is 1, 2 or 3 depending on the metal;

under suitable conditions to cause the formation of a coordinate bond between $[M(H_2O)_3(CO)_3]^{n+}$ and the tertiary amine nitrogen atom of the solid phase bound organic conjugate and thereby the release of the metal complexed agent thus formed from the support.

The linker may or may not be present. It may be already present in the available solid support or can be introduced later. When it is present it is preferably a good activating group for nucleophilic attack at C and selected from the group consisting of phenyl, vinyl, aryl and other non-aliphatic or aliphatic groups. The phenyl, vinyl, aryl, other non-aliphatic or aliphatic group may be substituted, and if they are they are preferably substituted with an electron withdrawing group selected from OR, R, NR_2, wherein R is an aliphatic or an arylic group.

In a preferred embodiment the linker is as shown in formula II:

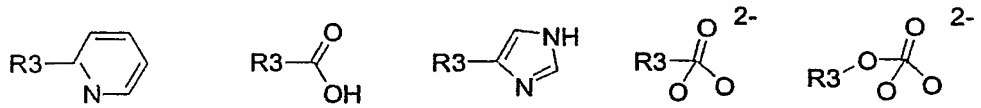


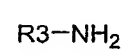
(II)

wherein X1 is C or O and X2 an electron withdrawing substituents and preferably a $-OCH_3$ group.

When R1 and R2 or one of R1 and R2 are non-coordinating organic groups they may be selected from alkyl, phenyl or benzyl or derivatives thereof.

Preferably, R1 and/or R2 are selected from the group consisting of





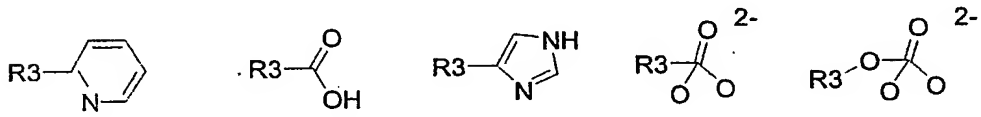
wherein R3 is directly the tertiary amine or is an aliphatic chain containing between 1 and 3 carbons.

The metal M may be any metal and is preferably selected from the group consisting of Tc, Re, Ru, Rh, Ir, Cu and Pt. The metal is most preferably ^{99m}Tc, ^{186}Re or ^{188}Re.

Preferably the metal is suitable for use as an imaging agent, e.g. by transmission of high-energy particles or paramagnetic characteristics, or as a radionuclide.

$[M(H_2O)_3(CO)_3]^{n+}$ can be generated by any suitable means known in the art. Suitable means for generating $[M(H_2O)_3(CO)_3]^{n+}$ are for example from the permetallate form as disclosed by Alberto et al. (J. Am. Chem. Soc. **123**, 3135-3136 (2001)) or in WO98/48848 (Alberto et al.).

The molecule according to formula I without the solid support is called herein the ligand. The ligand can be in particular a tridentate ligand if R1 and R2 are selected from the group consisting of



$R3-NH_2$

but also a bidentate chelator if R1 and R2 or one of both are an aliphatic or aromatic non-coordinating group.

The ligands used according to the invention in combination with $[M(H_2O)_3(CO)_3]^{n+}$ can be a diversity of tridentate ligands, the main requirement being the presence of a tertiary amine group as the central part of the ligand, which forms the C-N bond that is coupled with the solid support and cleaved upon complex formation. Preferably the ligands used are those based on aliphatic or aromatic amines or carboxylates and combinations thereof as donors. The ligand can also be a bidentate chelator if R1 and R2 but not both is

a non-coordinating aliphatic or aromatic group. This is shown in Example 10.

In particular diethylene triamine, picolylamine-*N*-acetic acid, *N*-(2-aminoethyl)-glycine or imino-diacetic acid can be used as ligands in the invention.

The ligand can be covalently linked to the solid support by first forming a halogenated resin e.g. by the methods described by Ngu and Patel (Tet. Lett. 38, 973 - 976 (1997). This halogenated resin can subsequently be reacted with a protected ligand. After deprotection, the ligand bound resin is obtained. If the ligand is attached to the solid phase by this method the covalent bond attaching it to the solid support will be a C-N bond. The ligand can also be synthesized on the solid support starting from an amino resin, e.g. by reductive amination and/or alkylation with alkyl halides as described in examples 5, 6, 8 and 9. Preparation of a loaded resin is discussed in more detail in examples 1-9.

In this specification the term ligand refers to a compound comprising at least one metal coordinating atom capable of forming a coordinating bond with a metal to form a stable metal-ligand complex. A ligand comprising more than one metal coordinating atom may be referred to as a chelator or a multidentate ligand. Bidentate ligands are ligands with two metal-coordinating atoms, tridentate ligands are ligands with three metal-coordinating atoms and tetradentate ligands are ligands with four metal coordinating atoms.

The biologically active molecule (also called herein "biomolecule") that may be coupled to the ligand can be any molecule that is active in diagnosis or therapy. The molecule can be coupled at any position except at the nitrogen linked

to the solid support. It may be a targeting molecule for directing the radioactive product to the site that needs to be diagnosed or treated or it may have a therapeutic activity that is independent from the radiolabel. The biologically active molecule may be selected from the group consisting of amino acids; steroids; proteins, in particular structural proteins, enzymes or antibodies; carbohydrates; polysaccharides and oligosaccharides; nucleosides, nucleotides, oligonucleotides and polynucleotides; lipids, peptides and pharmaceutically active small molecules such as central nervous system receptor binding compounds.

The biomolecule can be linked to the ligand with any suitable means known in the art e.g. by reductive amination of an aldehyde to a primary amine group of the ligand or by introducing a binding site at the arylic system. The biomolecule can be linked to the ligand prior to or after binding the ligand to the solid support.

It was found that the choice of the solid support may further improve the efficiency of the process of the invention. The solid support has to be able to swell in water, it has to be stable at reaction conditions, and it must not contain metal coordinating units. This is in particular the case when the solid support is a polyethylene glycol resin, or a hybrid of polyethylene glycol and polystyrene, e.g. a polystyrene resin with polyethylene glycol spacers with a benzyl alcohol anchoring group.

The process of the invention may further comprise the step of collecting the metal complexed agent (i.e. the radiopharmaceutical) for further use.

After preparation of n.c.a. ^{99m}Tc radiopharmaceutical, the solid phase polymer can be collected, washed and reused.

Preferably, the process is performed at a pH that is in the range of about 6.0-11.0, preferably in the range of about 7.5-9.5.

Suitable temperatures for performing the reaction are within the range of about 40-100°C. Preferably the reaction is performed in the range of about 70-82°C.

According to a further aspect thereof the invention relates to the solid phase bound ligand-biomolecule conjugate of formula I, and compositions comprising such a compound. Preferably these compositions are in a form, which can be stored during prolonged time periods under pharmaceutically acceptable conditions.

With the process according to the invention metal complexed ligand-biomolecule conjugates can be obtained with a high specific activity by filtration without further post-labeling purification.

According to a further aspect thereof the invention relates to the metal complexed ligand-biomolecule conjugates obtainable with the process according to the invention. Usually the conjugates are comprised in a composition which is the result of the process of the invention, and which is characterized in that it is essentially free of uncomplexed ligand-biomolecule conjugate e.g. the level of uncomplexed ligand-biomolecule conjugate in the composition containing them is in the 10^{-7} M range. Compositions with such characteristics are a further aspect of the invention.

Due to the short half-life of some isotopes used in

radiopharmaceuticals, e.g. ^{99m}Tc, labeling of ligand-biomolecule conjugates just prior to their use can be important for the specific activity of the complexed conjugate. The amount of decayed complex in a freshly labeled composition will be lower compared to the situation when the conjugate was complexed a substantial amount of time prior to its use.

Therefore, in yet a further aspect the invention relates to a kit for the preparation of a diagnostic or therapeutic pharmaceutical composition, comprising a container with the molecule of formula (I), in which the reaction with a solution of $[M(H_2O)_3(CO)_3]^{n+}$ take place. The container can be a vessel or column. The solution of $[M(H_2O)_3(CO)_3]^{n+}$ is introduced into the vessel or column to start the reaction. The solution can be part of the kit or provided by other means. In an alternative embodiment, the reagents for the preparation of the metal carbonyl $[M(H_2O)_3(CO)_3]^{n+}$ are comprised in the kit. In addition, the kit may comprise a facility for filtration.

The use of a kit further provides flexibility to the metal complex that can be formed since a selection of a suitable metal can be made just prior to the complexation reaction.

The principle of the preparation of no carrier added (n.c.a.) metal complexed compounds according to the invention is explained in **Figure 1**. A tridentate ligand e.g. diethylene triamine is bound via a linker, here a benzyl derivative, to a solid phase via a tertiary amine. To the chelator (ligand) a biomolecule, is attached, thus forming a ligand-biomolecule conjugate. Upon introduction of $[Tc(H_2O)_3(CO)_3]^+$, complex

formation occurs and the tridentate ligand replaces two aqua ligands. The remaining hydroxy ligand on $[Tc(H_2O)_2(OH)(CO)_3]^+$ can now attack the activated methylene group to induce C-N-bond cleavage. Activation of the methylene group occurs by complexation of the tertiary amino group to the technetium center, which withdraws electron density from the chelator and make it susceptible for nucleophilic attack.

The main species with reactivity towards the tertiary amine atom of the solid phase bound biomolecule-ligand conjugate is $[M(OH)(H_2O)_2(CO)_3]$. In solution $[M(OH)(H_2O)_2(CO)_3]$ is in equilibrium with the conjugate in the form of $[M(H_2O)_3(CO)_3]^{n+}$ and further dissociated forms, depending on the pH of the solution. It will be understood that, depending on the pH, $[M(OH)(H_2O)_2(CO)_3]$ is at least partially interchangeable with these species due to the equilibrium.

The invention is further explained with the following non-restrictive examples. In the Examples reference is made to the following figures:

Figure 1: The principle of the preparation of no carrier added (n.c.a.) metal complexed compounds according to the invention.

Figure 2: Mechanism of complex formation.

Figure 3: pH dependence of the cleavage reaction.

Figure 4: Temperature dependency of cleavage yield.

Figure 5: Reaction schemes of Examples 1, 2, 3, 4 and 10.

Figure 6: Reaction schemes of Examples 5, 6, 7, 8 and 9.

Figure 7: Reaction schemes of Examples 11, 12 and 13.

Figure 8: Structural formulas of the compounds of Example 14 and Table 1.

Figure 9: Structural formulas of the compounds with biologically active molecules.

EXAMPLES

EXAMPLE 1

A model ligand was covalently attached to an appropriate solid phase resin. The solid phase resin has to swell in water to allow diffusion of the Tc-species, and the anchoring group to which the ligand is coupled has to be an activating group for nucleophilic attack. The polystyrene/ polyethylene glycol resin TentaGel S AC (Rapp Polymere GmbH, Tübingen, Germany) fulfills these requirements. Its active site, a benzyl alcohol derivative, was converted into the corresponding bromide **1** (Ngu and Patel, Tet. Lett. 38, 973 - 976 (1997)).

N,N''-Bis(1-(4,4-dimethyl-2,6-dioxocyclohexyliden) ethyl)diethylenetriamine (**2**) (101.8 mg, 235.9 µmol) was dissolved in DMF (5 ml), resin **1** (196.6 mg, 47.2 mol) was added, and the mixture was gently stirred at room temperature for 22 hours. The reaction mixture was filtered, and the resin was washed with DMF (3 times), DMF and methanol (3 times alternating), and DMF (5 times). The protecting groups were removed by washing the resin 10 times with a solution of hydrazine hydrate, 2% in DMF (1 ml) for 5 minutes. The reaction mixture was filtered, and the resin was washed with DMF (3 times), DMF and methanol (5 times

alternating), DMF (3 times), and diethyl ether (3 times). The resin was dried at high vacuum to give product 3 in a yield of 192.3 mg (97.8%; capacity 0.236 mmol/g, coupling efficiency 100%).

Free amino groups on the solid phase resins were visualized by color tests: bromophenol blue solution in water for alkaline resins and trinitrotoluenesulfonic acid (TNBS) in DMF/diisopropylethylamine 10:1 exclusively for primary amines. Resin 3 was positive in both tests, whereas the protected intermediate was negative on TNBS staining. The capacity of the resins (in mmol of bound chelator per gram) and the efficiency for the coupling of the chelators to the solid supports were calculated from the N-content of the resins as determined by elementary analyses.

The chelating capacity of resin 3 was verified by stirring it in a 1mM solution of $[^{99}Tc(H_2O)_3(CO)_3]^+$ (7 equivalents) at room temperature. Analyzing of the filtrated solution by β^- liquid scintillation counting showed a decrease of activity of 14%, which is consistent with quantitative complex formation on the resin. HPLC analyses exhibited only peaks of the starting material, indicating that no cleavage from the solid phase occurred under these mild conditions. The once formed ^{99}Tc-complex turned out to be stable under the conditions used for labeling. Even prolonged heating at 80°C for 5 hours in phosphate buffer pH 7.5, yielded only 3% beta-activity in the solution, at least one order of magnitude lower than expected.

EXAMPLE 2

An other tridentate ligand, yielding non charged complexes, was attached to the same resin as in Example 1. *N*-Picolylamine acetic acid ethyl ester (54 mg, 280 µmol) was dissolved in DMF (3 ml) and resin **1** (280 mg, 67 µmol) was added. The mixture was gently stirred at room temperature for 15 hours, the reaction mixture was filtered, and the resin was washed with DMF (3 times), DMF and methanol (3 times alternating), and diethyl ether (3 times). The protected intermediate was positive on bromophenol blue and negative on TNBS staining. The protecting groups were removed by gently stirring the resin in a mixture of water (5 ml) and sodium hydroxide 1M (0.30 ml) for 28 hours. Filtration of the resin, washing with DMF (3 times), DMF and methanol (3 times alternating), DMF (3 times), and diethyl ether (3 times) and drying at high vacuum gave product **4** in a yield of 268 mg (96%; capacity 0.209 mmol/g, coupling efficiency 87.1%). Resin **4** was negative on all of the staining reactions.

EXAMPLE 3

An other tridentate ligand, yielding negatively charged complexes, was attached to the same resin as in example 1. Dimethylimino diacetate hydrochloride (6.4 mg, 33 µmol) and diisopropylethylamine (11.2 µl, 66 µmol) were dissolved in DMF (0.5 ml) and resin **1** (280 mg, 67 µmol) was added. The mixture was gently stirred at room temperature for 24 hours, the reaction mixture was filtered, and the resin was washed with DMF (3 times), DMF and methanol (3 times alternating), methanol and water (3 times alternating). The protected intermediate was positive on bromophenol blue and

negative on TNBS staining. The protecting groups were removed rinsing the resin with aqueous NaOH (0.1M for 3 hours, then 0.01M for 12 hours). Filtration of the resin, washing with NaOH 0.1M (2 times), water (5 times), water and methanol (3 times alternating), methanol (3 times), and diethyl ether (3 times) and drying at high vacuum gave product 5 in an yield of 35 mg (100%; capacity 4 mmol/g, coupling efficiency 18%). Resin 5 was negative on all of the staining reactions.

EXAMPLE 4

Another tridentate ligand, yielding non-charged complexes, was attached to the same resin as in example 1.

N^5-tert-butyloxycarbonyl)-5-amino-3-azapentane acid ethyl ester(74 mg, 280 μmol) was dissolved in DMF (3 ml) and resin 1 (300 mg, 72 μmol) was added. The mixture was gently stirred at room temperature for 15 hours, the reaction mixture was filtered, and the resin was washed with DMF (3 times), DMF and methanol (3 times alternating), and diethyl ether (3 times). The protected intermediate was positive on bromophenol blue and negative on TNBS staining. The ethyl ester group was removed by gently stirring the resin in a mixture of water (5 ml) and sodium hydroxide 1M (1.40 ml) for 28 hours. Filtration of the resin, washing with DMF (3 times), DMF and methanol (3 times alternating), DMF (3 times), and diethyl ether (3 times) and drying at high vacuum gave the Boc protected acid. The Boc group was removed by stirring the resin in a mixture of TFA and DCM (1:1) for 5 minutes, filtration, and by stirring the resin in a mixture of TFA and DCM (1:1) again, now for 10 minutes. Washing as described above gave product 6 in a yield of 72 mg (96%, capacity 22

mmol/g, coupling efficiency 94%). Resin 6 was positive on TNBS staining.

EXAMPLE 5

NovaSyn TG resin has an aliphatic amino anchoring group. In this example, the synthesis of the chelator picolylamineacetic acid on the solid support is described.

NovaSyn TG resin (100 mg, 30 umol)and pyridine-2-carbaldehyde (14.3 µl, 150 µmol) were stirred in methanol (3 ml) at room temperature for 20 hours. The reaction mixture was filtered, and the resin was washed with DMF (3 times), and DMF and methanol (3 times alternating). Sodium triacetoxy- borohydride (31.8 mg, 150 µmol) in DMF (2 ml) was added to the resin. After stirring at room temperature for 5 hours, the reaction mixture was filtered, and the resin was washed with DMF (4 times) and methanol (3 times). $NaHCO_3$ (10% in water) was added to the resin. After 3 hours, the resin was washed with DMF (3 times), water (3 times), ethanol (3 times), and diethyl ether (3 times) and dried to give the aminopyridine intermediate which was positive on bromophenol blue and slightly positive on TNBS staining.

A mixture of bromoacetic acid ethyl ester (16.6 µl, 150 µmol) and diisopropyl ethylamine (5.1 µl, 30 µmol) in ethanol (2.5 ml) was added to the resin. After stirring at room temperature for 24 hours, the reaction mixture was filtered, and the resin was washed with ethanol (5 times) and dried. NaOH (1 M) was added to the resin to remove the ethyl ester protecting group. After one day stirring at room temperature, the reaction mixture was filtered, and the resin was washed with water (5 times), ethanol (3 times) and diethyl

ether (2 times) and dried to give **8** in a yield of 86.4 mg (83.7%; capacity 0.10 mmol/g, coupling efficiency 43%).

EXAMPLE 6

NovaSyn TG resin (100 mg, 30 µmol), ethyl bromoacetate (33.2 µl, 300 µmol) and diisopropylethylamine (12.9 µl, 75 µmol) were reacted as described in the second part of example 5 to give **9** in a yield of 96.2 mg (93%).

EXAMPLE 7

NovaSyn TGT resin has a hydroxytrityl anchoring group. The hydroxy group was converted to the chloride as described (J.M.J Frechert et al., Tetrahedron Lett. 1975, 3055).

Dimethyliminodiacetate hydrochloride (16.2 mg, 82 µmol) and diisopropylethylamine (21 µl, 123 µmol) were dissolved in DMF (2 ml). Chlorinated Novasyn TGT resin (41 µmol) was added. The coupling reaction as well as the ester hydrolyses were done as described in example 3 with the exception that diisopropylethylamine (14 µl, 82 µmol) was added after 3 hours reaction time. Product **10** was obtained in a yield of 132 mg (81%; capacity 0.015 mmol/g, coupling efficiency 6%).

EXAMPLE 8

NovaSyn TGR resin has an aminomethyl anchoring group with two aryl substituents on the methylene group. Resin **11** was prepared analogous to the synthesis of **8** described in Example 5.

NovaSyn TGR resin (166 mg, 30 µmol) was reacted with the same amount of reagents as in example 5 to give **11** in a yield of 153 mg (90 %; capacity 0.08 mmol/g, coupling efficiency 44%).

EXAMPLE 9

Resin **12** was prepared analogous to the synthesis of 9 described in Example 6.

NovaSyn TGR resin (166 mg, 30 µmol) was reacted with the same amount of reagents as in example 6 to give **12** in a yield of 142.2 mg (84%).

EXAMPLE 10

In this example, a bidentate chelator is attached to Tentagel S AC bromide **1**.

N,N'-Dimethylethylenediamine (110 µl, 1040 µmol) was dissolved in DMF (1 ml), resin **1** (108.6 mg, 26 µmol) was added, and the mixture was gently stirred at room temperature for 24 hours. The reaction mixture was filtered, and the resin was washed with DMF (3 times), DMF and methanol (3 times alternating), water (3 times), DMF and isopropanol (3 times alternating), water (3 times, iospropanol (3 times), and diethyl ether (3 times). The resin was dried at high vacuum to give product **13** in a yield of 101.6 mg (98.1%; capacity 0.24 mmol/g, coupling efficiency 100%). Resin **13** was positive on bromophenol blue and negative on TNBS staining.

EXAMPLE 11

In this example, the synthesis of a conjugate yielding a bioactive complex, which intercalates into double

stranded DNA is described. The bioactive unit, a pyrene derivative, is attached to the chelating unit on the solid support.

N-Boc-*N''*-Dde protected diethylene triamine was coupled to **1** as described in the preparation of **3**. The bis-protected intermediate was positive on bromophenol blue and negative on TNBS staining. The Dde protecting group was removed by stirring the resin in hydrazine hydrate (1.5 ml, 2% in DMF) five times for 10 minutes, followed by filtration. Positive staining with TNBS confirmed the removal of the Dde protecting group. The pyrene group was introduced by reductive amination. 1-Pyrenaldehyde (28.5 mg, 120 μmol) and methanol (4 ml) were added to the mono-deprotected resin (103 mg, 24 μmol), and the mixture was stirred at room temperature for 20 hours. After filtration and washing with DMF, sodium triacetoxyborohydride (25 mg, 120 μmol) in DMF (5 ml) was added, and the mixture was stirred for 24 hours at room temperature. Washing with DMF and methanol (as above) gave the Boc-protected resin bound pyrene diethylenetriamine derivative which was positive on bromophenol blue and negative on TNBS staining. Finally, the Boc protecting group was removed by stirring the resin in trifluoroacetic acid (50% in CH_2Cl_2) for 5 minutes, followed by filtration and another treatment with trifluoroacetic acid (50% in CH_2Cl_2) for 10 minutes. Washing (as described above) gave product **7** (capacity 0.18 mmol/g, coupling efficiency 82%). Resin **7** was positive on bromophenol blue and on TNBS staining.

EXAMPLE 12

In this example, biotin (Vitamin H) is attached to the chelating unit. In contrast to example 11, the chelator/biomolecule-conjugate is synthesized prior to the binding to the solid support.

Triethylenetetramine (0.150 ml, 1.00 mmol) was dissolved in THF (30 ml), and the solution was cooled to -78°C. A solution of ethyl trifluoroacetate (0.109 ml, 1.00 mmol) in THF (5 ml) was added within 30 min at -78°C, and the solution was stirred at that temperature for 4 hours. Then it was warmed up to 0°C.

In the meantime, (+)-Biotin (244 mg, 1.00 mmol) in DMF (8 ml) was heated to 80°C to give a colorless solution. N,N'-Dicyclohexylcarbodiimide (216 mg, 1.05 mmol) and N-hydroxysuccinimide (121 mg, 1.05 mmol) was added to the hot solution. The mixture was allowed to slowly cool down to room temperature. A white powder precipitated. Stirring was continued for 4 hours.

The two mixtures were mixed at 0°C and stirred for 30 minutes to give a white gel. The THF was evaporated to give a white suspension. After stirring at room temperature for 18 hours, the solvent was removed *in vacuo* and water was added to the residue. The pH was adjusted to 3-4. The mixture was filtered, the eluate was neutralized and the water was removed *in vacuo*. The crude product was purified by column chromatography (silica, dichloromethane/methanol/triethylamine 5:1:0.1) to give N-biotinyl-N'''-trifluoroacetyl-triethylenetetramine in a yield of 60.5 mg (0.129 mmol, 12.9%). The structure was confirmed by mass spectroscopy and NMR.

N-Biotinyl-N'''-trifluoroacetyl-triethyltetramine (40.3 mg, 86 µmol), triethylamine (1.8 µl, 17 µmol) and resin 1 (71.7 mg, 17 µmol) were reacted as described in example 1. The TFA-resin was dried at high vacuum to give product **14** in an yield of 67 mg (85%, capacity 3 mmol/g, coupling efficiency 13%). Resin **14** was positive on bromophenol blue and negative on TNBS staining. Attempts to remove the TFA protecting group with sodium carbonate (10 % in water) failed according to negative results upon TNBS staining.

EXAMPLE 13

In this example, a method for the preparation of labeled peptide derivatives is described. A protected dipeptide with a free carboxylic end group was coupled to a partially protected polyamine. All of the protecting groups were removed, and Dde protection was introduced to selectively block the primary amino groups at the peptide and the chelator. This allowed to selectively bind the conjugate to a solid support via formation of a tertiary amino group from one of the unprotected secondary amino groups of the chelator.

N,N',N''-tri(*tert*-butyloxycarbonyl)triethylene-tetramine (103.3 mg, 234.5 µmol), Boc-Phe-Gly-OH (75.6 mg, 234.5 µmol), PyBOP (183 mg, 352 µmol) and diisopropyl ethylamine (20 µl, 117 µmol) were dissolved in dichloromethane (2.5 ml). The solution was stirred at room temperature for 4 hours. Periodically, diisopropylethylamine (20 µl, 117 µmol) was added to keep the pH >7. The solvent was removed *in vacuo*, the residue was dissolved in ethyl acetate and washed in brine (3 times), cold HCl 0.5M (3 times), $NaHCO_3$ 10% (2 times), and brine (3 times). The organic phase was dried over $MgSO_4$, and

the solvent was removed *in vacuo* to give *N*-(*tert*-butyloxycarbonyl-phenylalanyl-glycyl)-*N'*,*N''*,*N'''*-tri(*tert*-butyloxycarbonyl)-triethylenetetramine in an yield of 175.2 mg (231.6 µmol, 98.8%). The structure was confirmed by mass spectroscopy and NMR.

The Boc protecting groups were removed by stirring the product (162.2 mg, 0.169 mmol) in HCl in ethyl acetate (ca. 1 M, 3 ml). After stirring at room temperature for 9 hours, the solvent was removed *in vacuo*. The residue was dissolved in water and stirred for 1 hour (pH was 2-3), then NaOH (1 M, 313 mmol) was added to neutralize the solution, and the solvent was evaporated *in vacuo* to give H-Phe-Gly-NH-C_2H_4-NH-C_2H_4-NH-C_2H_4-NH_2 in quantitative yield. The structure was confirmed by mass spectroscopy and NMR.

H-Phe-Gly-NH-C_2H_4-NH-C_2H_4-NH-C_2H_4-NH_2 (113 mg, 0.152 mmol) and 2-Acetyldimedone (Dde-OH) (70.0 mg, 0.335 mmol) were dissolved in ethanol (4 ml). After stirring at room temperature for 20 hours, analysis by TLC exhibited full conversion of the amine to a single product. The solvent was removed to give Dde-Phe-Gly-NH-C_2H_4-NH-C_2H_4-NH-C_2H_4-NH-Dde. The structure was confirmed by mass spectroscopy. The crude product was used without separation from the surplus of 2-acetyldimedone.

Dde-Phe-Gly-NH-C_2H_4-NH-C_2H_4-NH-C_2H_4-NH-Dde (76 µmol) and resin 1 (79.2 mg, 19 µmol) were reacted as described in example 1. The protected intermediate was positive on bromophenol blue and negative on TNBS staining. The Dde protecting groups were removed as well as described in example 1, to give product **15**. Resin **15** was positive on bromophenol blue and on TNBS staining.

EXAMPLE 14

Labeling conditions: $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was prepared out of $[^{99m}TcO_4]^-$ using a boroncarbonate kit (Alberto et al, *J. Am. Chem. Soc.* 123, 3135-3136 (2001)). 1 mg of the solid-phase bound chelators (0.2 mmole) were given to the $[^{99m}Tc(H_2O)_3(CO)_3]^+$-solution (1 ml), the mixtures were shortly sonificated and then heated to 82°C for 30 minutes. The solutions were separated from the solid phase resin by filtration and analyzed by HPLC with gamma-detection.

With all of the solid phase bound chelators, formation of soluble complexes was observed. The yield varied on chelator type and reaction conditions between 5 to 50% (Table 1).

EXAMPLE 15

Resins 3 and 4 were also labeled in a one pot procedure, combining the formation of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ and the cleavage reaction. 1 mg of solid-phase bound chelators (0.2 mmole) was added to a boroncarbonate kit (Mallinckrodt Medical, Petten, the Netherlands; Alberto et al., J. Am. Chem. Soc. 123, 3135-3136 (2001)). $NaTcO_4$ as eluted from a generator was added to the vial, and the mixture was kept at 78°C to 82°C for 20 to 60 minutes. The pH was 11. Cleavage yield was between 8 and 32%, conversion of pertechnetate between 40 and 54%.

EXAMPLE 16

For the labeling reactions on resin 4, reaction conditions such as pH value and reaction temperature were varied to find optimal reaction conditions. For use as a

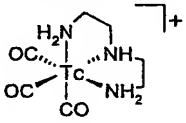
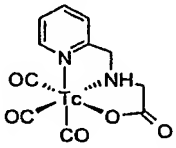
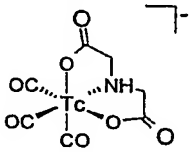
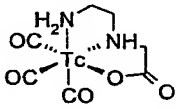
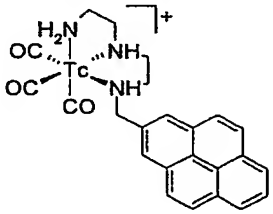
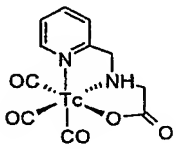
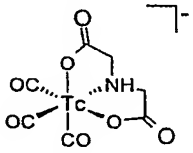
radiotracer, complete conversion of the starting material $[^{99m}Tc(H_2O)_3(CO)_3]^+$ and formation of one single product is required. Purification steps after the labeling procedure have to be avoided because of the radioactivity of the samples and their rapid decay ($t_{1/2}$ = 6.2 hours). High cleavage yields are desirable to get solutions of high radioactivity.

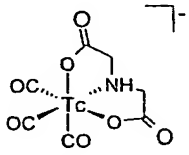
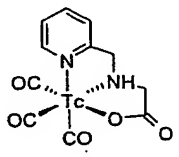
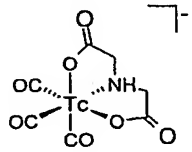
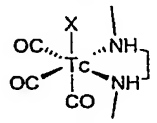
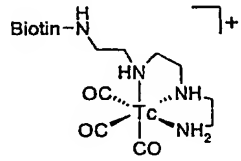
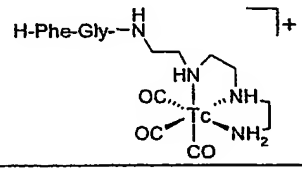
pH dependence of the cleavage reaction is shown in Figure 3. Cleavage yield increases from pH 6 with a maximum at pH 8.5. This is in consistence with deprotonation of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ to $[^{99m}Tc(OH)(H_2O)_2(CO)_3]$ (pK_s for the Rhenium analog: 7.5; Egli et al, *Organometallics* 16, 1833-1840 (1997)) and, therefore, with the theory that a Tc-coordinated hydroxy ion is the nucleophile which attacks the CH_2-group to cleave the C-N bond. Increasing the pH to 11 reduces the cleavage yield again. This could be due to formation of the negatively charged species $[^{99m}Tc(OH)_2(H_2O)(CO)_3]^-$ which reduces the electrophilicity of a coordinated amino group.

Temperature dependence of the reaction of resin 4 with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was studied by analyzing the reaction products after full conversion of $[^{99m}Tc(H_2O)_3(CO)_3]^+$. At room temperature, only complex formation at the resin occurred, the solution after filtration from the solid phase exhibited no radioactivity. At 43°C, cleavage yield was observable but low. Then, it increased with increasing temperature (**Figure 4**). This clearly shows that there is a competition between complex formation at the resin and the cleavage reaction, with the cleavage reaction having the higher activation energy barrier. However, very high temperatures could be a disadvantage in view of the stability of the solid phase resin and of attached

biomolecules. A reaction temperature of 70°C to 82°C is preferred for resin 4.

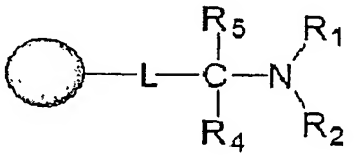
Table 1

solid-phase bound chelator (resin)	cleaved ^{99m}Tc-complex	cleavage yield	conditions
3 (TentaGel)		27%	1 hour 82°C
4 (TentaGel)		52% 28%	1 hour 82°C 2 hours 56°C
5 (TentaGel)		20%	1 hour 82°C
6 (TentaGel)		22%	1 hour 82°C
7 (TentaGel)		5%	1 hour 82°C
8 (NovaSyn TG)		8%	1 hour 82°C
9 (NovaSyn TG)		11% 4%	1 hour 82°C 3 hours 56°C

10 (NovaSyn TGT)		93% 79%	1 hour 82°C 3 hours 56°C
11 (NovaSyn TGR)		53% 15%	1 hour 82°C 3 hours 56°C
12 (NovaSyn TGR)		83% 78%	1 hour 82°C 3 hours 56°C
13 (TentaGel)		10%	1 hour 82°C
14 (TentaGel)		14%	1 hour 82°C
14 (TentaGel)		13%	2 hours 82°C

CLAIMS

1. Process for generating a metal complexed agent, comprising contacting (I) a solid phase bound organic conjugate represented by the formula



(I)

wherein:

the sphere is the solid phase;

C is a methylene group that may be substituted by one or two groups R4 and R5, which can be in particular aliphatic or aromatic substituents, or RO, RS or R_2N, wherein R is an aliphatic or arylic group,

L is a linker that may or may not be present, that is coupled to the solid support and has activating properties towards nucleophilic attack to the C group and is preferably a phenyl, alkyl, allyl or aryl; and

R1 and R2 are the same or different and are a metal coordinating group or a non-coordinating organic group,

which solid phase bound organic conjugate is optionally derivatized at one or both of R1 and R2 with a biologically active molecule,

with (II) $[M(H_2O)_3(CO)_3]^{n+}$,

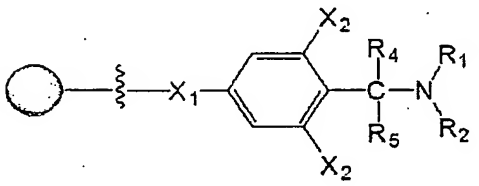
wherein M is selected from the group consisting of technetium (Tc), rhenium (Re), rhodium (Rh), platinum (Pt), iridium (Ir)

and copper (Cu) and n is 1,2 or 3 depending on the metal; under suitable conditions to cause the formation of a coordinate bond between $[M(H_2O)_3(CO)_3]^{n+}$ and the tertiary amine nitrogen atom of the solid phase bound organic conjugate and thereby the release of the metal complexed agent thus formed from the support.

2. Process according to claim 1, wherein the linker is selected from the group consisting of phenyl, vinyl, aryl, and other non-aliphatic and aliphatic groups.

3. Process according to claim 2, wherein the phenyl, vinyl, aryl or other non-aliphatic and aliphatic groups are substituted with an electron withdrawing group selected from OR, R, NR_2, wherein R is an aliphatic or arylic group.

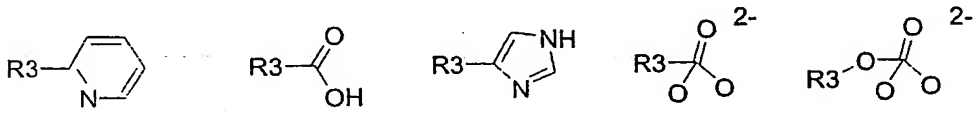
4. Process according to claims 2, wherein the linker is as shown in formula II:



(II)

wherein X1 is C or O and X2 an electron withdrawing substituents and preferably a $-OCH_3$ group.

5. Process according to claim 1, wherein R1 and/or R2 are selected from the group consisting of



6. Process according to claim 1, wherein R1 and R2 are an aliphatic or aromatic substituent, such as $-CH_3$, C_2H_5 or $CH_2C_6H_5$.

7. Process according to claim 1, wherein M is selected from the group consisting of Tc, Re, Ru, Rh, Ir, Cu and Pt.

8. Process as claimed in claim 7, wherein the metal is selected from the group consisting of ^{99m}Tc, ^{186}Re and ^{188}Re.

9. Process according to claim 1, wherein the biomolecule is selected from the group consisting of amino acids; steroids; peptides; proteins, in particular structural proteins, enzymes or antibodies; carbohydrates; polysaccharides and oligosaccharides; nucleosides, nucleotides, oligonucleotides and polynucleotides; lipids, peptides and pharmaceutically active small molecules such as central nervous system receptor binding compounds.

10. Process according to claim 1, wherein the solid phase support is a polyethylene glycol resin, or a hybrid of polyethylene glycol and polystyrene, e.g. a polystyrene resin with polyethylene glycol spacers with a benzyl alcohol anchoring group.

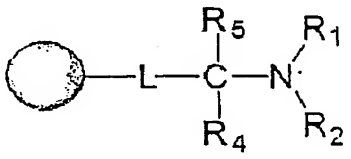
11. Process according to claim 1, further comprising the step of collecting the metal complexed agent for further use.

12. Process according to claim 1, wherein the process is performed at a pH that is in the range of about 6.0-11.0, preferably in the range of about 7.5-9.5.

13. Process according to claim 1, wherein the process is performed at a temperature in the range of about 40-100°C, preferably in the range of about 70-82°C.

14. Process according to claim 11, which further comprises bringing the collected metal labeled conjugate into a pharmaceutically acceptable form.

15. A solid phase bound organic conjugate represented by the formula



(I)

wherein L, C, R1, R2, R4 and R5 are as defined in claim 1.

16. A solid phase bound organic molecule according to claim 15, characterized in that the biologically active molecule is selected from the group consisting of amino acids; steroids; peptides; proteins, in particular structural proteins, enzymes or antibodies; carbohydrates; polysaccharides and oligosaccharides; nucleosides, nucleotides, oligonucleotides and polynucleotides; lipids, peptides and pharmaceutically active small molecules such as central nervous system receptor binding compounds.

17. A solid phase bound organic molecule according to claim 15, wherein the solid phase support is a polyethylene glycol resin, or a hybrid of polyethylene glycol and polystyrene, e.g. a polystyrene resin with polyethylene glycol spacers with a benzyl alcohol anchoring group.

18. A solid phase bound organic molecule according to claim 15 as depicted in Table 1.

19. A metal complexed organic molecule obtainable by

the process according to claim 1.

20. A kit for the preparation of a diagnostic or therapeutic pharmaceutical composition, comprising a container with the molecule of formula (I), in which the reaction with a solution of $[M(H_2O)_3(CO)_3]^{n+}$ can take place.

21. Kit as claimed in claim 20, wherein the container is a vessel or column.

22. Kit as claimed in claim 20, further comprising a solution of $[M(H_2O)_3(CO)_3]^{n+}$.

23. Kit as claimed in claim 20, further comprising the reagents for the preparation of the metal carbonyl $[M(H_2O)_3(CO)_3]^{n+}$.

24. Kit as claimed in claim 20, further comprising a facility for filtration.

Figure 1

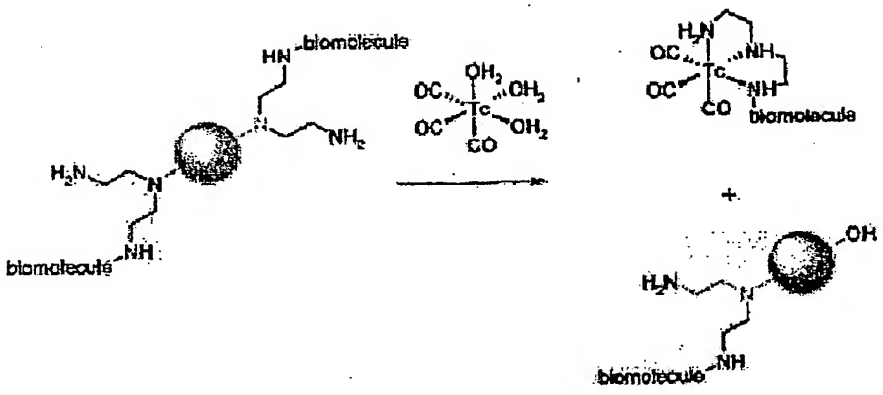

biomolecule
HN
NH2
H2N
N
NH
biomolecule
OH2
OC
Tc
CO
OH2
OC
CO
H2N
NH
OC
Tc
NH
OC
CO
biomolecule
+
OH
H2N
N
NH
biomolecule

Figure 2

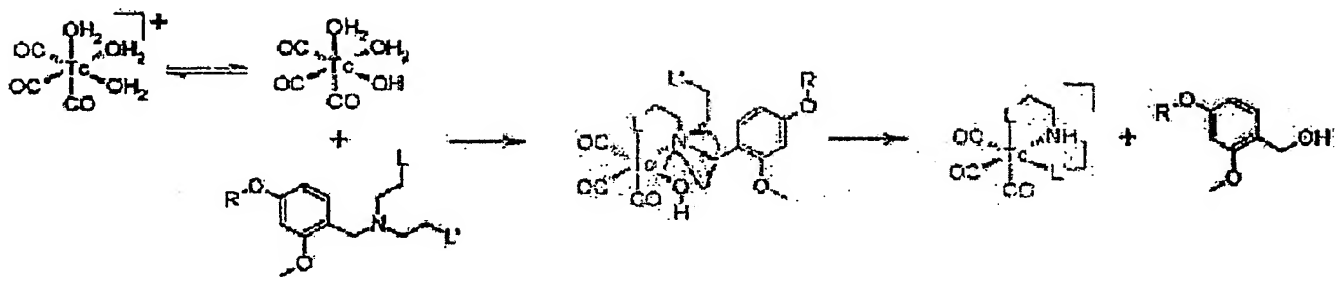


Fig. 3

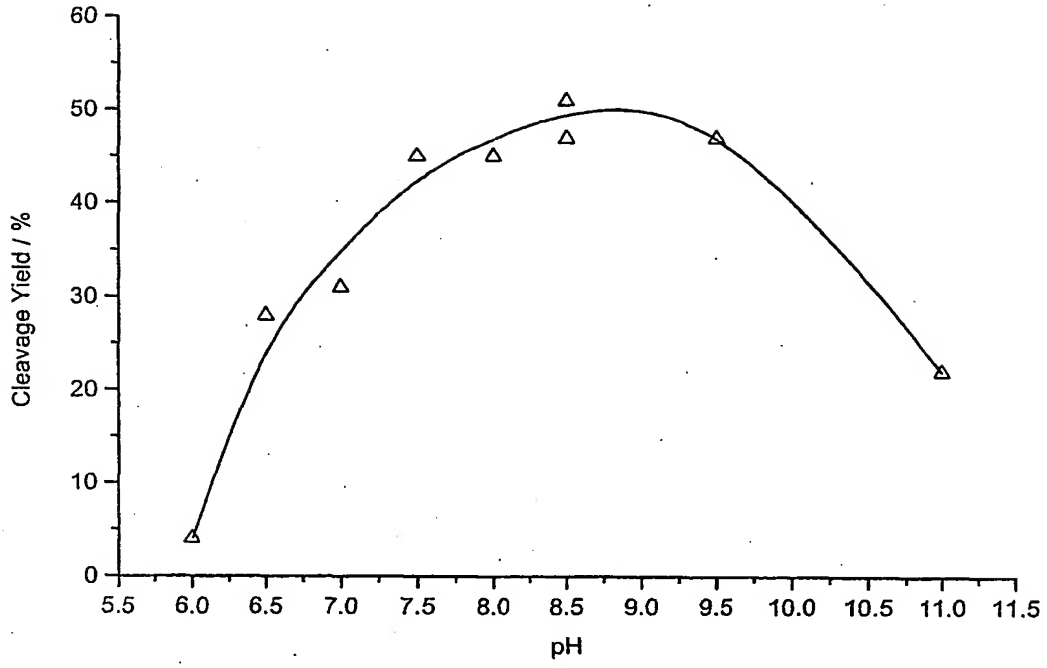

Cleavage Yield / %
60
50
40
30
20
10
0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
pH

Fig. 4

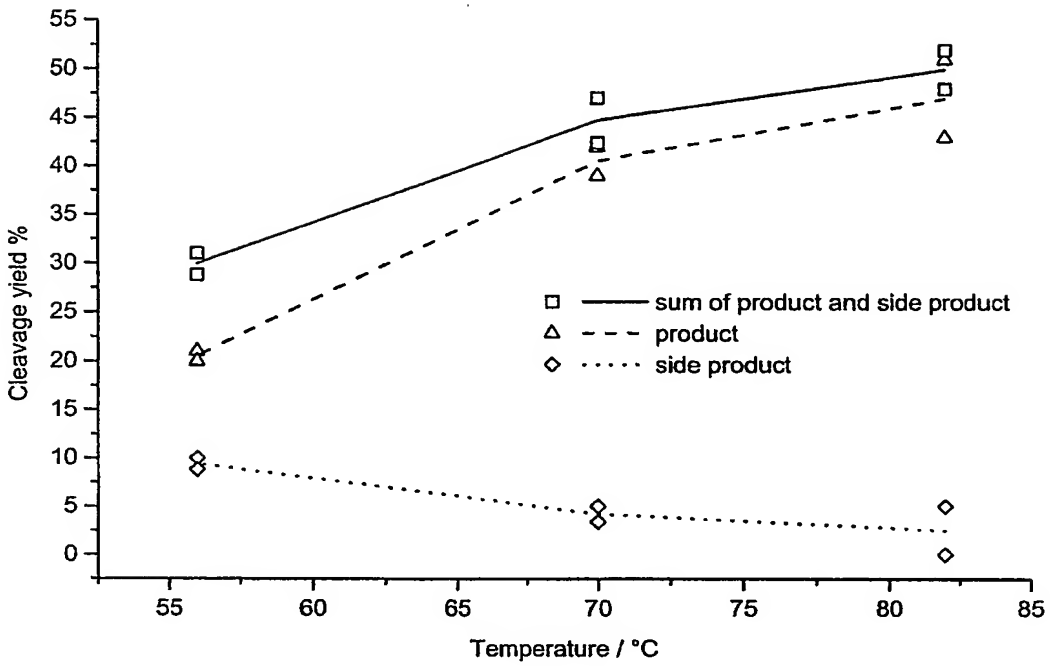

Cleavage yield %
55
50
45
40
35
30
25
20
15
10
5
0
55
60
65
70
75
80
85
Temperature / °C
sum of product and side product
product
side product

Fig.5

Examples with TentaGel resins

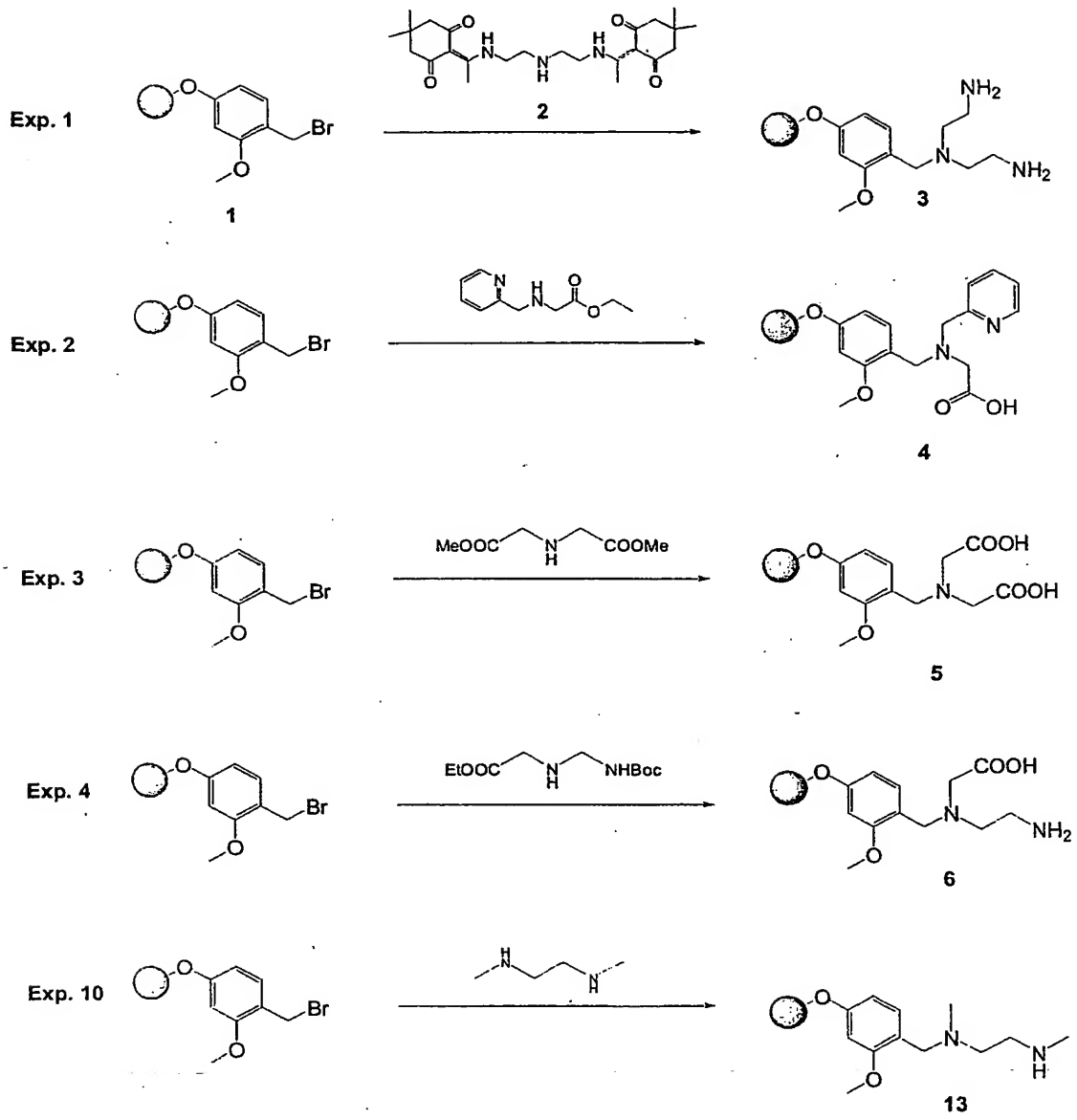


Fig. 6

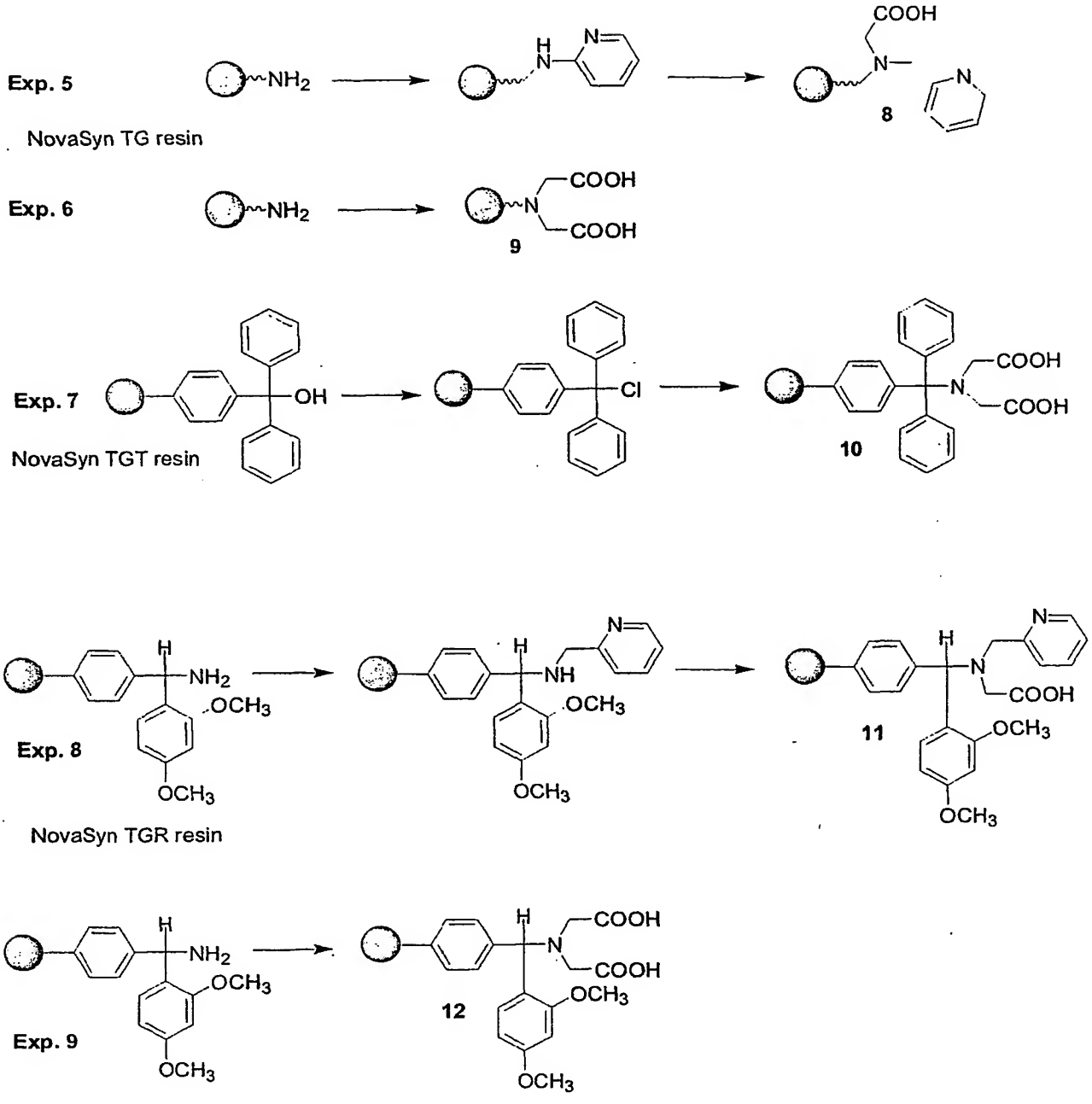
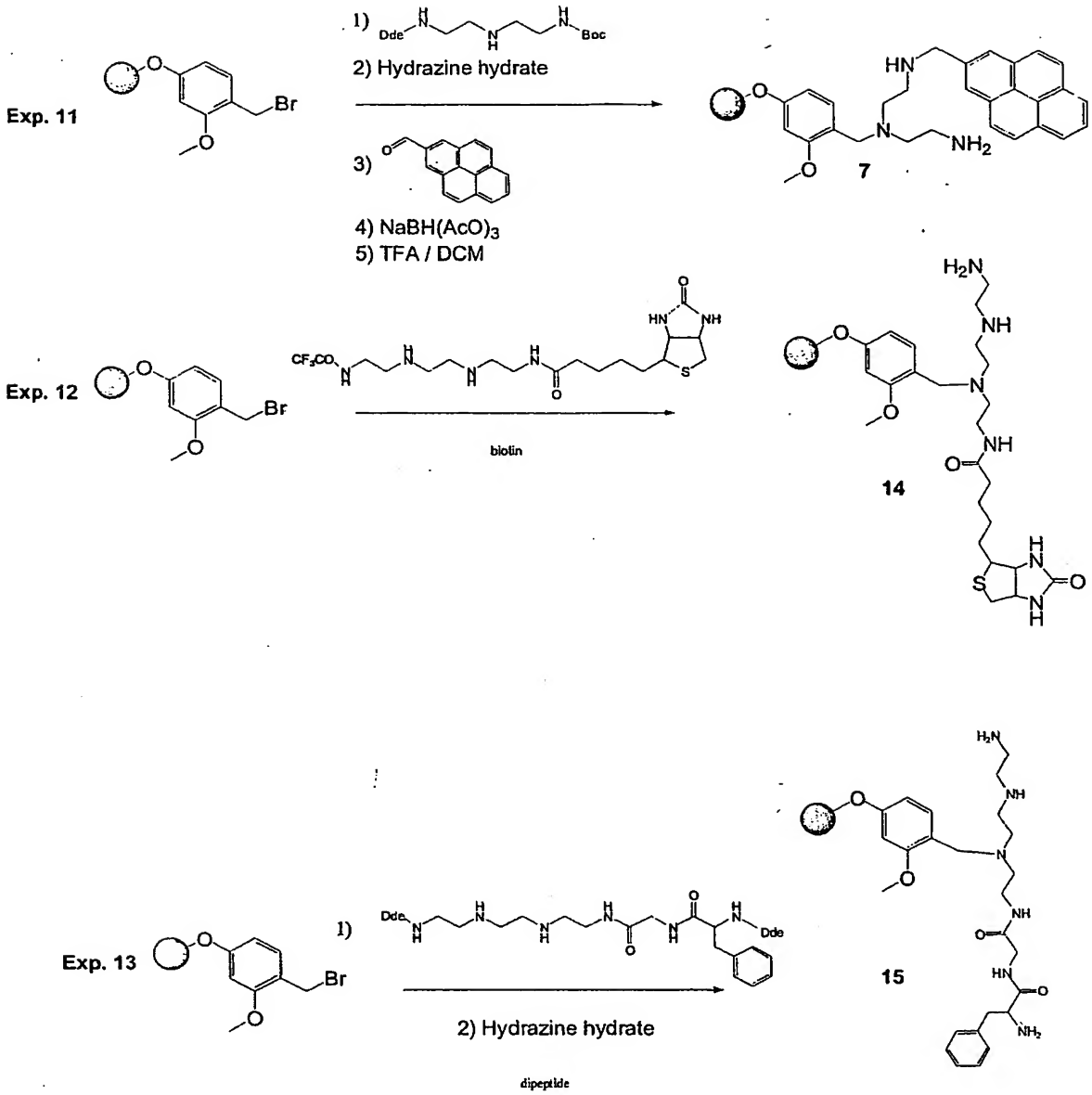

Exp. 5
NovaSyn TG resin
8
Exp. 6
9
Exp. 7
NovaSyn TGT resin
10
Exp. 8
NovaSyn TGR resin
11
Exp. 9
12

Fig.7


Exp. 11
1)
Dde
Boc
2) Hydrazine hydrate
3)
4) NaBH(AcO)3
5) TFA / DCM
7
Exp. 12
biotin
14
Exp. 13
1)
Dde
2) Hydrazine hydrate
dipeptide
15

Example 14 and Table 1

Fig.8

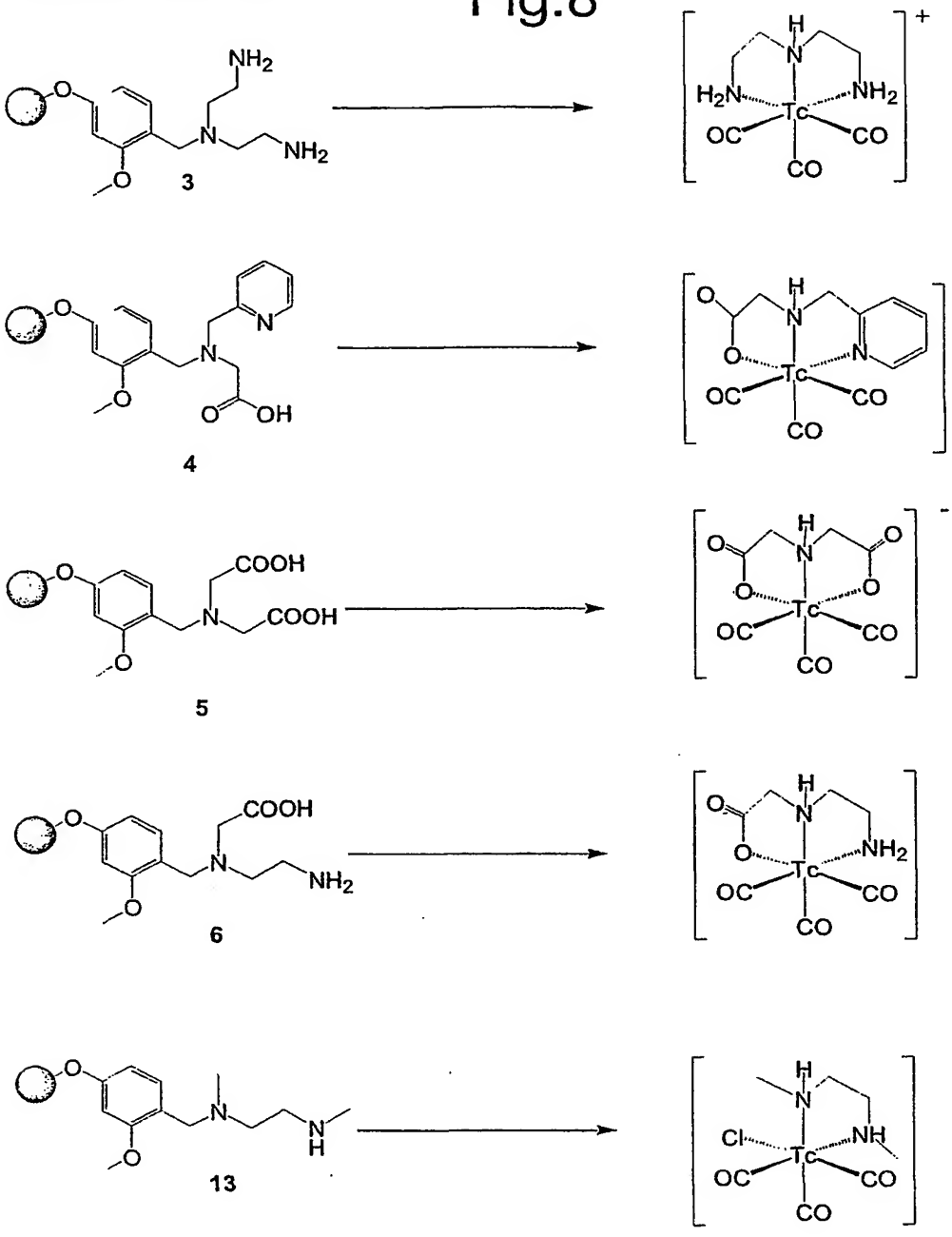
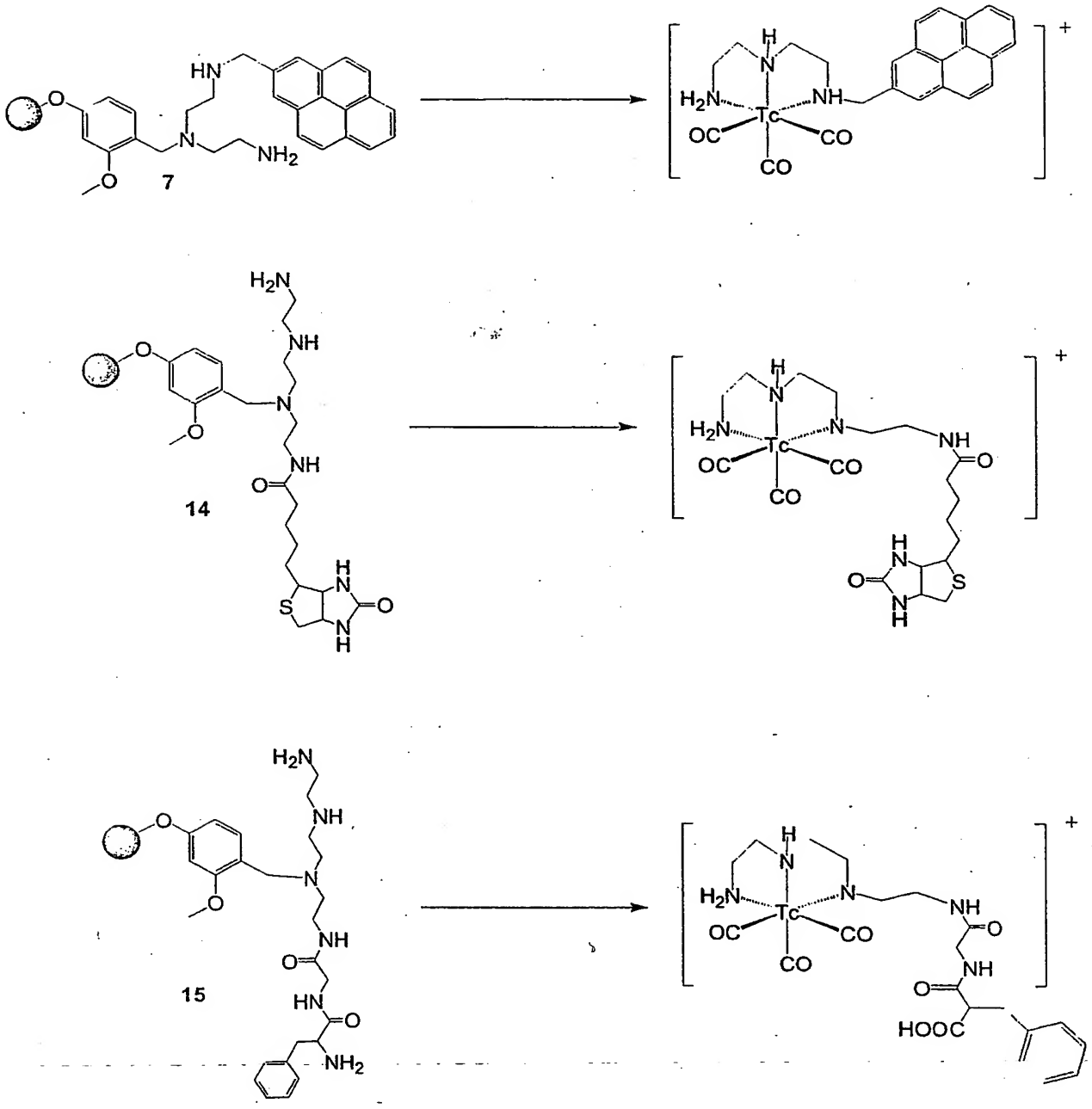


Fig. 9



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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number
WO 2004/022105 A3

(51) International Patent Classification[7]: A61K 51/04, 51/12, C07F 1/08, 13/00, 15/00

(21) International Application Number:
PCT/US2003/027665

(22) International Filing Date:
2 September 2003 (02.09.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02078743.8 3 September 2002 (03.09.2002) EP

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
22 April 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PREPARATION OF M(CO)3-COMPLEXES BY SOLID PHASE TECHNIQUES VIA METAL ASSISTED CLEAVAGE FROM THE SOLID SUPPORT

(57) Abstract: The present invention relates to a process for generating a water soluble metal complexed agent, comprising contacting a solid phase bound organic conjugate represented by the formula I with $|M(H_2O)_3(CO)_3|^{n+}$, under suitable conditions to cause the formation of a coordinate bond between $|M(H_2O)_3(CO)_3|^{n+}$ and the tertiary amine nitrogen atom of the solid phase bound organic conjugate and thereby the release of the metal complexed agent thus formed from the support. The invention further relates to the conjugate of formula (I) and to a kit for performing the process.

WO 2004/022105 A3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 03/27665

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K51/04 A61K51/12 C07F1/08 C07F13/00 C07F15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data

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Date of the actual completion of the international search	Date of mailing of the international search report
10 December 2003	11/02/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Elliott, A

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